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Evidence for stanniocalcin binding activity in mammalian blood and glomerular filtrate

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Background. The 50 kD form of the hormone stanniocalcin-1 (STC50) is widely distributed in organs such as kidney, lung, and liver. Kidney collecting duct cells produce STC50 for local targeting to proximal tubule cells to increase phosphate reabsorption. As such the current dogma is that in most organs STC50 is a purely local mediator that is not released into the circulation. However, liver hepatocytes contain high levels of both STC50 and its receptor but little evidence of STC production, suggesting that the hormone may in fact be delivered to hepatocytes systemically. Moreover, previous data suggest that red blood cells may in fact bind STC. In this report, we have sought to identify STC binding activity in mammalian blood.

Methods. Human, pig, and dog red blood cells were analyzed in STC receptor binding assays. Mouse red blood cells and adult mouse kidney were also analyzed histologically for the presence of STC ligand and receptor.

Results. Saturable, high affinity STC receptors were identified on red blood cells from all species. More intriguingly, STC binding activity was also identified in glomerular filtrate, indicative of a soluble, filterable STC binding protein. This binding protein was subsequently observed being reabsorbed in proximal straight tubules.

Conclusion. These findings suggest that our inability to detect STC in mammalian serum is due to its being attached to soluble and tethered forms of a high-affinity binding protein. This could be a means of delivering STC to distant targets as well as a mechanism for removing unwanted hormone from the circulation.

One of the more intriguing facets of stanniocalcin-1 (STC-1) physiology relates to the nature of the circulating hormone. The perspective from our laboratory for some time has been that STC only circulates in females and only during pregnancy and lactation. This conclusion is based on the fact that STC has proven to be undetectable in the serum of humans and other mammals by radioimmunoassay. Serum from males and nonpregnant females is generally incapable of causing significant displacement of the radioiodinated tracer. We have also been unable to extract significant amounts of STC from serum using either concanavalin A or immunoaffinity chromatography [1]. During pregnancy and lactation, however, circulating STC is readily detectable in both rats and mice [2, 3]. Because the appearance of STC in the serum coincides with a massive up-regulation in ovarian STC gene expression, the circulating hormone is believed to originate in the ovaries. In support of this notion, Western blot analysis has recently shown that this circulating STC is an 84 kD variant, identical in size to the smallest of three big STC variants that are synthesized and secreted by ovarian theca cells [3].

Big STC is produced however by only a few select organs; steroidogenic cells in the ovaries and adrenal glands [4, 5]. The other form of STC which is perhaps better known is the 50 kD disulfide-linked homodimer and structural homologue of fish STC [6]. Recently designated as STC50 to distinguish it from big STC, it also appears to be the more widely distributed form of the hormone. STC50 is abundant in tissues such as liver, kidney, lung, and spleen, and the experimental evidence to date suggests that it likely functions locally in each of these organ systems as a paracrine mediator [7]. The analysis of STC50 clearance kinetics has shown that a 100 μg hormone bolus becomes nondetectable by radioimmunoassay within minutes of injection into male and female rats. On the other hand, when the same analysis is conducted with radiolabeled tracer, STC50 has values of distribution ($T_{1/2}\alpha$; 1 minute) and clearance ($T_{1/2}\beta$; 60 minutes) that are typical for a polypeptide hormone [1]. These findings led us to conclude that STC50 was being rapidly metabolized after entering the serum to the extent that it was being rendered nonimmunoreactive, and that the slower clearing tracer was likely fragmented hormone. At the time, this seemed to be a plausible means of eliminating from the circulation what was believed to be a purely paracrine mediator. However, one intriguing observation

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that was not examined in greater detail was the extent to which the tracer hormone was bound up and then released by red blood cells. In fact, the dissociation curve for ^{125}I-STC50 from the red blood cell fraction mirrored that of its disappearance from the plasma [1], suggesting that red blood cells were perhaps capable of binding and releasing STC50 in accordance with prevailing tracer levels.

Another facet of STC50 physiology that has so far defied explanation relates to the liver. Northern blot analysis has revealed that liver tissue has very low levels of STC gene expression. This is true in the case of both embryos and adults [8, 9]. And yet, for a tissue with such a low level of endogenous gene expression, immunocytochemistry and Western blot analysis indicates that hepatocytes contain surprisingly high levels of STC50, most of which is in the mitochondrial fraction [1]. Similarly, hepatocyte membranes and mitochondria both contain relatively high levels of saturable, high-affinity (K_D 0.2 nmol/L) STC receptors [7]. Collectively, these data suggest that hepatic STC50 may not in fact be locally derived, but is perhaps instead transported there via the circulation. The purpose of this study therefore is to address the hypothesis that there is STC carrying capacity within the elements that constitute whole blood, which could account for its unusual abundance in organs such as the liver.

METHODS

Receptor binding studies on red blood cell ghosts

Red blood cell ghosts were isolated from dog, pig, and human whole blood using an established protocol [10]. Heparinized, fresh whole blood was centrifuged at 2000 × *g* for 10 minutes to pellet the red blood cells. The red blood cell pellet was then subjected to two 5-minute washes in phosphate-buffered saline (PBS), pH 7.6. Washed red blood cells were lysed with a hypotonic buffer (5 mmol/L sodium phosphate, pH 8.0). The resulting ghosts were pelleted by centrifugation at 27,000 × *g* and washed extensively in lysis buffer prior to use in receptor binding assays.

Saturation binding assays were carried out on purified red cell ghosts to obtain estimates of K_D and B_{max}. These assays were performed as previously described [7, 11], using a fusion protein of STC and human placental alkaline phosphatase as the experimental ligand (STC·AP) and human placental alkaline phosphatase (AP) as the control ligand, both of which were expressed in Madin-Darby canine kidney (MDCK) cells [7]. The binding assay has previously been employed to characterize STC receptors on microsomal membranes, mitochondria, nuclei, and lipid droplets from a variety of tissue types [3, 5, 7, 11]. Purified red cell ghosts were incubated in increasing concentrations of STC·AP or AP dissolved in Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin (BSA), pH 7.5, for 90 minutes at room temperature. To separate the bound and free ligand, samples were centrifuged for 2 minutes at 13,000 × *g*. The pellets were subsequently washed three times in HBSS containing 0.1% BSA, pH 7.5 (HBHA buffer), lysed, and heat-treated to destroy endogenous AP activity. An AP detection buffer was then added to all samples for the quantification of AP activity by spectrophotometry. Specific binding was determined by subtracting the AP activity in the ghosts incubated with AP alone from those incubated with STC·AP. By taking into account the specific activity of the STC·AP ligand, the data were then converted into units of STC for calculation of K_D and B_{max}.

Immunocytochemistry and in situ ligand binding studies

To localize STC and its receptor on red blood cells at the histologic level, immunocytochemical and in situ ligand binding studies were carried out on 5 micron tissue sections of mouse embryonic kidney and heart. Tissues were dissected from timed pregnant CD-1 mice, fixed by immersion in PBS containing 4% paraformaldehyde, and embedded in paraffin for sectioning.

Immunocytochemistry was performed on embryonic day 14.5 (E14.5) mouse kidney as previously described using a specific antiserum generated in rabbits against human recombinant STC50 (hSTC50) [1, 2]. Following an overnight incubation in primary antiserum (1:1000), the slides were washed extensively and the sites of antibody binding were visualized with biotinylated secondary antibodies and the Vectastain ABC peroxidase detection system (Vector Laboratories, Burlingame, CA, USA). Sections were subsequently counterstained with hematoxylin, dehydrated, and mounted.

In situ ligand binding was performed on E16.5 mouse heart and perfusion-fixed, adult mouse kidney [12] in an attempt to localize STC receptors and/or binding activity at the histologic level. The method has been previously characterized for specificity [7, 11] and employs the same STC·AP fusion protein and AP control protein as described above. The technique has been successfully used to localize STC receptors at the histologic levels in liver, kidney, ovary, and mammary gland [3, 7, 11]. Fixed, paraffin-embedded tissue sections were equilibrated in HBHA buffer and then incubated for 90 minutes in HBHA buffer containing 1 nmol/L STC·AP. Control slides were incubated in either AP alone or STC·AP containing 1 μmol/L rhSTC. Slides were then washed and processed for visualization of bound AP activity.

RESULTS

Receptor binding studies on red blood cells

The results of the receptor binding studies on human female red cell ghosts are shown in Figure 1. Figure 1A

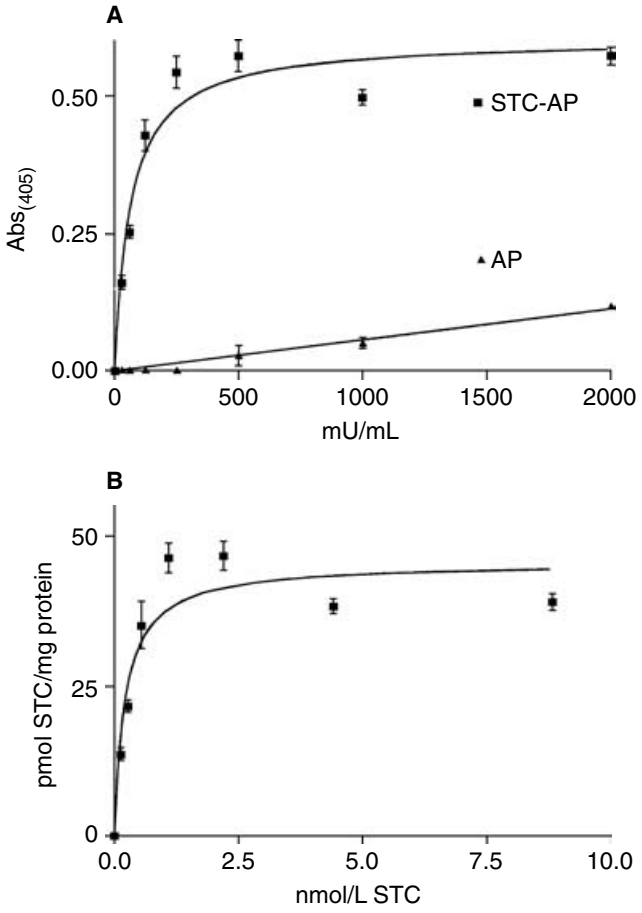

Fig. 1. Saturation binding assay on human red blood cell ghosts. Red blood cell ghosts were isolated from human blood (female) as described in the **Methods** section. The binding assay was performed using a fusion protein of stanniocalcin (STC) and human placental alkaline phosphatase (AP) as the experimental ligand (STC·AP) and human placental AP as the control ligand. (*A*) The raw binding data obtained with STC·AP and AP. (*B*) Specific STC·AP binding after transformation of the data. This assay yielded an estimated K_D of 0.2 ± 0.05 nmol/L and a B_{max} of 45.5 ± 2.3 pmol/mg protein (mean ± SEM).

shows the raw binding data obtained with STC·AP and AP, whereas Figure 1B shows specific STC·AP binding after transformation of the data. The results of this saturation binding assay yielded a K_D of 0.22 ± 0.05 nmol/L and a B_{max} of 45.5 ± 2.3 pmol/mg protein. A second estimate on the same sample yielded similar results (K_D of 0.63 ± 0.1 nmol/L and a B_{max} of 58.6 ± 5.2 pmol/mg protein). The results obtained with pig and dog red cell ghosts also revealed the presence of saturable, high-affinity binding sites. However, the estimates of K_D for nonprimate mammals were significantly higher than those obtained in humans. Saturation binding analysis of red cell ghosts from three male pigs yielded K_D and B_{max} estimates of 17 ± 2.2 nmol/L and 75 ± 5.1 pmol/mg protein, respectively (mean ± SEM) ($N = 3$). Similarly, binding studies on red cells from a single male dog specimen yielded a K_D of 9.2 ± 1.2 nmol/L and a B_{max} of 118 ± 10.8 pmol/mg protein. These receptor binding results have been summarized in Table 1.

Table 1. Stanniocalcin (STC) binding characteristics of mammalian red blood cells[a]

Species	K_D *nmol/L*	B_{max} *pmol/mg protein*
Human	0.2 ± 0.05	45.5 ± 2.3
Pig	17 ± 2.2	75 ± 5.1
Dog	9.2 ± 1.2	118 ± 10.8

[a]The data are represented as the mean ± SEM.

Immunocytochemistry and in situ ligand binding studies

The results of the histologic analysis are shown in Figure 2. Figure 2A shows the results obtained following in situ ligand binding. The panel shows a field of red blood cells within the ventricle of E16.5 mouse heart. Intense binding is evident over a subpopulation of red blood cells (red arrows), comprising ~10% of the cells within the field of view. In contrast, little or no binding is apparent to the remaining cells. Strong ligand binding is also evident over the ventricular trabeculae of the developing heart (black arrows). Ligand binding to the subset of red blood cells was specific to STC·AP as no staining was apparent when AP was used as the control ligand (inset, upper right). These findings therefore extended the receptor binding studies in Figure 1 by revealing that the ability to bind STC was confined to just a subset of red cells.

A similar proportion of red blood cells were positively stained for the presence of STC ligand. Figure 2B illustrates the immunocytochemical staining results obtained in E14.5 mouse kidney. The red arrow points to a group of red blood cells confined within a vein, a subfraction of which were intensely stained by the antiserum; others were weakly stained, and the remainder were unstained. These finding lent support to the in situ ligand binding results in Figure 2A, and collectively implied that a small fraction of the cells were positive for both ligand and ligand binding activity. The developing proximal tubules and collecting ducts also exhibited intense staining for STC as previously described [8]. Staining of an adjacent tissue section with antiserum preabsorbed overnight with STC (1 μg/mL) produced no staining of either the red blood cells or kidney tubules, thus demonstrating the specificity of the reaction (inset, upper right).

The presence of soluble STC binding activity in shown in Figure 2C, which is a low magnification image of the adult mouse kidney cortex following in situ ligand binding with STC·AP fusion protein (the kidney capsule is marked with a black arrow). A number of structures have been labeled in the image, including the collecting ducts and the distal convoluted tubules (red asterisks), both of which exhibit an intense, punctate cellular pattern of staining. Previous studies have shown that this

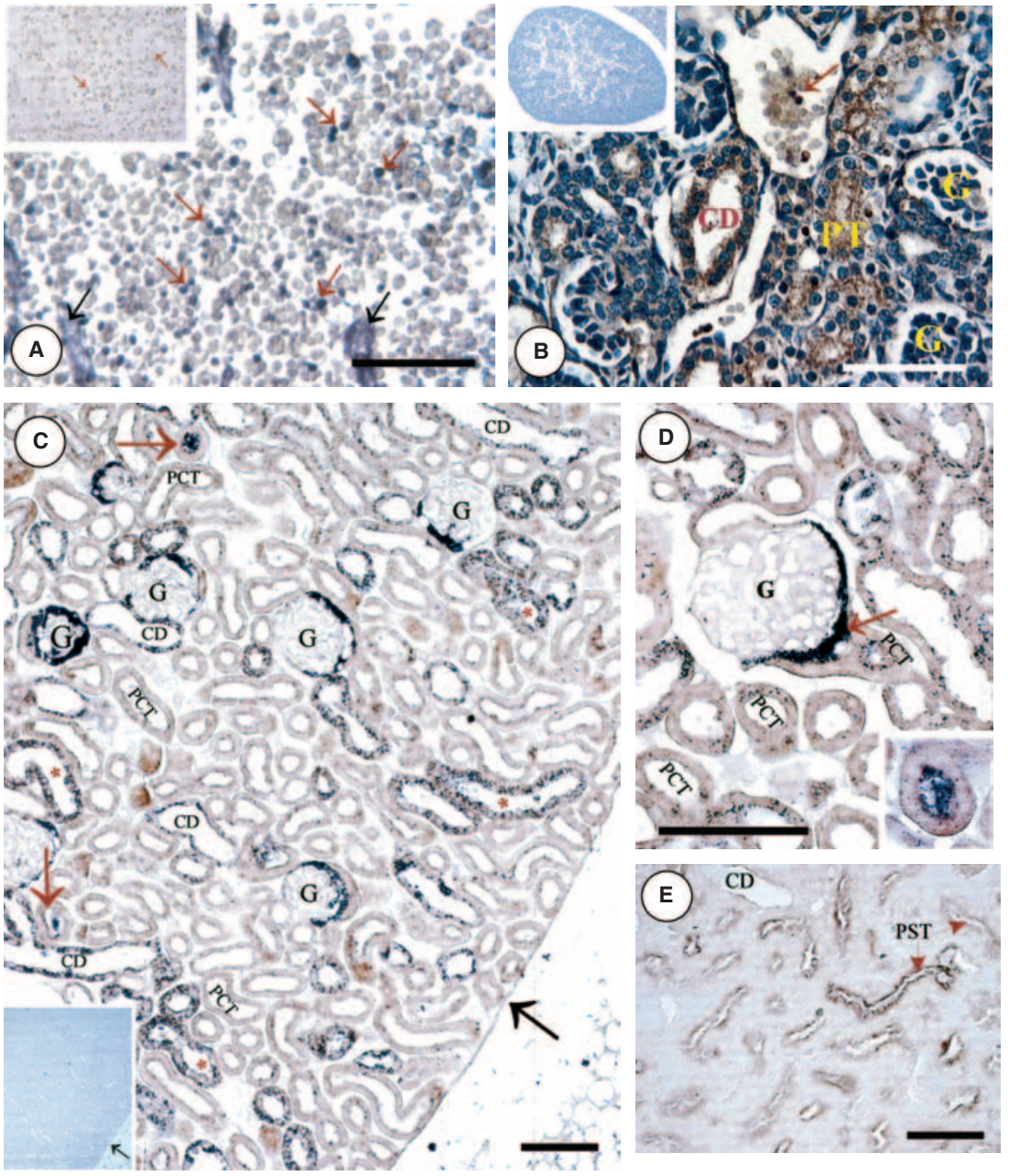

Fig. 2. In situ ligand binding and immunocytochemistry. (*A*) In situ localization of stanniocalcin (STC) binding sites on red blood cells in embryonic day 14.5 (E14.5) mouse heart. Intense binding is evident to a sub-population of red blood cells (red arrows). Strong ligand binding is also evident to the ventricular trabeculae (black arrows). Ligand binding was specific to STC·alkaline phosphatase (STC AP) as no staining was apparent when AP was used as the control ligand (inset, upper right). (*B*) Immunocytochemical localization of STC on red blood cells in E16.5 mouse kidney. The arrow points to a group of red blood cells, some of which are intensely stained; others are weakly stained or unstained. The developing proximal tubules and collecting ducts are also stained. Staining of an adjacent section with antiserum preabsorbed with STC produced no staining (inset, upper right). (*C*) In situ localization of STC binding activity in glomerular filtrate. Intense binding activity is evident around each glomerulus (G) in the region encompassed by Bowman's space that contains glomerular filtrate. Binding activity is also present in the lumen of some proximal convoluted tubules (red arrows). The collecting ducts (CD) and the distal convoluted tubules (red asterisks) also exhibited intense, punctate cellular staining. The use of AP as a ligand produced no specific staining (inset, lower left). (*D*) In situ localization of STC binding activity in the glomerulus. This higher magnification image shows that the majority of ligand binding activity was associated with the glomerular filtrate in Bowman's space and not Bowman's capsule, the glomerular capillaries or the mesangial cells. Proximal convoluted tubules (PCT) exhibited a sparser pattern of punctate staining. The inset shows intense STC binding activity within a proximal convoluted tubule lumen. (*E*) In situ localization of STC binding sites on proximal straight tubule luminal cell membranes (red arrows). The use of AP as a ligand produced no specific staining (not shown). The calibration bar equals 50 microns in all panels.

punctate staining is indicative of mitochondrial STC receptors within individual tubule cells [7]. In contrast, the majority of proximal convoluted tubules are only weakly stained. More important, however, is the intense binding activity that is associated with individual glomeruli, many of which are in evidence in Figure 2C. The ligand binding to the glomerulus was by far the most intense within the kidney and had the appearance of solid black staining deposits. Moreover, as the higher magnification of a single glomerulus in Figure 2D shows, this ligand binding activity was associated entirely with the fluid-filled Bowman's space, where the glomerular filtrate forms and collects prior to entering the nephron tubule. In contrast, there was no binding activity in evidence over the glomerular capillaries, associated mesangial cells, or for that matter, Bowman's capsule. It is also important to point out here that STC binding activity was not necessarily present in all glomeruli, and that in some specimens none was detected. However, we have repeatedly observed this phenomenon in both rat and mouse kidney.

Clues as to the fate of the soluble STC binding activity are apparent in Figure 2C and D. The red arrows in Figure 2C highlight two proximal convoluted tubules in which binding activity is visibly evident within the tubule lumen. A higher magnification image has been inset in Figure 2D showing the intense binding activity that is associated with the luminal filtrate and luminal brush border of individual tubule cells. These findings are indicative of the passage of this soluble binding activity through the nephron. However, none of this binding activity is evident in the most distal nephron segments shown in Figure 2C; namely the distal convoluted tubules (asterisks) and collecting ducts. The possible reason for this is illustrated in Figure 2E, which shows a field of proximal straight tubules (S_3 segment) near the cortical-medullary junction. In these tubule segments from both rats and mice, intense ligand binding was found to be associated strictly with the luminal cell membranes. As such, these findings were indicative of a reabsorptive mechanism whereby the soluble binding activity was being taken up by S_3 segment tubular cells. Of further significance in this respect, we have obtained no evidence of filtrate binding activity in any nephron segments downstream of the proximal straight tubules (loop of Henle, thick ascending limb, etc.) in either rats or mice.

DISCUSSION

The results of the present study have revealed that mammalian red blood cells have significant carrying capacity for the hormone STC. In humans and domestic mammals we have obtained unequivocal evidence for the presence of high-affinity STC binding sites on purified red blood cell membranes. In humans, the values obtained for K_D were not unlike those obtained for membrane-associated STC receptors from rodent liver and kidney [7]. In contrast, the estimates of K_D obtained in domestic animals were at least tenfold higher, but it remains to be seen if this has physiologic significance. What may be physiologically significant, however, is that the capacity for STC binding was appreciably higher on red blood cells than most other tissues that have been examined to date. For instance, liver, kidney, ovary, and adrenal membranes [5, 7, 11] have binding capacities in the low picomole range (1 to 10 pmol/mg protein). The lone exception is the adipocyte, which has a sixfold greater membrane binding capacity than that observed in most other cells (64 pmol/mg) and extraordinarily high receptor levels on the lipid droplet membrane (1200 pmol/mg), where both STC and its receptor are heavily sequestered [5]. Hence, fat cell membranes approximate red blood cells in terms of binding capacity for STC.

The high binding capacity of red blood cells could be reflective of a specific role in the transport of STC to tissues that would otherwise have no access to the hormone. There is in fact a precedent for this in the case of growth hormone, which is instead sequestered inside the erythrocyte. This has been viewed as either a means of removing abnormally high levels of growth hormone from the serum or of delivering growth hormone to cells peripheral to its site of synthesis [13]. Two examples where the latter scenario could certainly apply in the case of STC are liver hepatocytes and fat cells, both of which contain STC receptors and readily detectable levels of cellular STC, but very low levels of endogenous STC production [5, 9, 14]. Red blood cells also possess receptors for insulin, insulin-like growth factor-1 (IGF-1), prolactin, transforming growth factor-β (TGF-β), and leukemia inhibitory factor, to name only some [15–19], and their purpose can vary. In the case of insulin, ligand binding activates a Na^+/Mg^{2+} exchanger to increase the levels of Mg^{2+} within the cells [17]. As such, we should not rule out the possibility that similar mechanisms might be operative in the case of STC and red blood cell ion transport, especially in view of its well-known role as regulator of transepithelial calcium and phosphate transport in kidney and gut [6, 20].

The discovery of specific binding within the glomerular filtrate adds an entirely new dimension to our current concept of STC signaling. It implies that a soluble binding protein circulates in mammalian blood and is freely filtered by the kidneys. Given the molecular weight cut-off within the glomerular capillaries, its overall mass would have to be no greater than ~70 kD. The data further suggest that following its filtration, the binding protein passes through the proximal convoluted tubules and is then completely reabsorbed by the proximal straight segment. Here it is presumably metabolized by tubular cells. One unresolved aspect of the glomerular filtrate binding

activity, however, is that it was not observed in all of the kidneys which were examined, male or female. Therefore, the physiologic conditions that lead to its presence need to be established. It would also appear that the ligand accompanies the binding protein during the filtration process, as subsequent studies have revealed that on occasions when the glomerular filtrate does contain STC binding activity, it also contains visible STC immunoreactivity as revealed by immunocytochemistry (unpublished observations). Therefore, the soluble binding protein could be a means of clearing STC from the serum.

Collectively, our findings provide plausible explanations as to why STC is generally undetectable in mammalian serum and why bolus injections of STC50 become undetectable by immunoassay so soon thereafter [1]. In light of the present findings it would now appear that in both circumstances, much of the hormone is likely sequestered by binding proteins that mask all antibody epitopes. As such, these findings now raise several important questions, one of which relates to the types of STC-1 (STC50 and big STC) that are bound by these proteins under normal physiologic conditions. Given that the ligand used in our binding studies was a fusion protein of STC50 and AP they can obviously bind STC50. However, it remains to be seen if big STC [4] can also bind with the same high affinity. A second question posed by these findings is whether or not the binding capacity within red blood cells is subject to regulation under conditions such as pregnancy and lactation, when STC (the big STC variant) is detectable in the serum by radioimmunoassay [2]. During pregnancy and lactation, large quantities of big STC are delivered to and sequestered by the mammary glands via the bloodstream [3]. Thus, it is possible that the soluble and/or tethered forms of the binding protein are down-regulated at this time to allow for systemic delivery of the hormone. Finally, although the binding protein on red blood cells may very well represent a mechanism for delivering STC to distant targets, it is equally possible that the soluble form is a mechanism for removing unwanted hormone from the circulation, albeit via renal clearance as opposed to endothelial cell sequestration, as has proven to be the case for the atrial natriuretic factor clearance receptor [21].

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